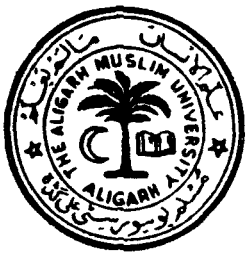

STUDIES ON THE PHYSIOLOGICAL RESPONSES INDUCED BY THE TREATMENT OF CERTAIN CHEMICALS IN THE SEEDS AND SEEDLINGS

DISSERTATION

SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE AWARD OF THE DEGREE OF

Master of Philosophy

IN

BOTANY

SHAMSUL HAYAT

LABORATORY OF PLANT PHYSIOLOGY
DEPARTMENT OF BOTANY
ALIGARH MUSLIM UNIVERSITY
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1993

DEDICATED

TO

MY PARENTS

Dr. AQIL AHMAD
M. Sc., Ph. D. (Alig.)
PD. Res. Train., DANIDA (Denmark)

BOTANY DEPARTMENT
ALIGARH MUSLIM UNIVERSITY
ALIGARH-202002

Dated: 26-10-93

CERTIFICATE

This is to certify that **MR. SHAMSUL HAYAT** has worked under my supervision for the M.Phil degree in Botany. He has fulfilled all conditions required to supplicate the M.Phil degree. I, therefore, approve that he may submit his dissertation entitled **"Studies on the physiological responses induced by the treatment of certain chemicals in the seeds and seedlings"**.

(AQIL AHMAD)
Supervisor of Research

ACKNOWLEDGEMENT

I bow in reverence to 'Allah' whose benign benediction gave me the required zeal and confidence to complete this task.

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(SHAMSUL HAYAT)

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CHAPTER - 1

I N T R O D U C T I O N

CHAPTER - 1

INTRODUCTION

The establishment of the next generation very much depends on the successful germination of the seed, therefore, it is supposed to be the most precarious stage in the life cycle of the plants. However, at a time when the seed is in a state of high metabolic activity where the embryo is dependent on the endosperm, cotyledons or nucellus for the supply of respiratory substrate, a sufficient amount of these precious organic and inorganic solutes is lost into the atmosphere around the seed. Though, this phenomenon is purely passive (Simon and Harun, 1974) taking place only from the outermost layers of the cells of hydrated seeds but the loss might be putting the embryo under stress at these initial stages of germination when the solubilization of stored food has just started. The presence of the pores in the phospholipids at a moisture content below 20% (Luzzati and Husson 1962) allow the escape of these substances. However, the rate of the loss of solutes is known to decrease with time as the water level in the seed goes above 20%. This leakage is considered to be a measure to provide an index of seed viability because the seeds with low-viability or non-viable lose larger quantities of the substances than highly viable seeds.

Out of the many other factors, the most positively correlated with low seedling vigour in the leakage of sugars, amino acids and electrolytes from the hydrated seeds (Becwar et al. 1982). This enrichment of the atmosphere around the seed enables it to sustain higher rate of growth of seed pathogens (Simon, 1978). In addition to this, the loss of vigour in imbibing legume seeds may be due to the destruction of seed tissues and the establishment of infection sites by massive cellular rupture occurring during the seed hydration.

The furnishment of the lamellar bilayer to establish normal physiological control system of the cell membrane, on the leakage, to become operative is largely regulated by water level which must be above 30% (Wilkins, 1987). However, the role played by the metabolic state of the seed and the hormonal balance cannot be denied.

Keeping this in mind, it is proposed to study the following aspects in the germinating seeds of pea (Pisum sativum var. Boneville and Arkel).

i) To assess the native values of different components, to be estimated.

ii) the pattern of leakage and its relationship with the metabolic state of the cotyledons and embryo, under varied conditions.

iii) a regulatory role of certain exogenously applied hormones on the leakage and seed metabolism under similar conditions as in the previous experiments will be approached.

CHAPTER - 2

REVIEW OF LITERATURE

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REVIEW OF LITERATURE

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CHAPTER - 2

REVIEW OF LITERATURE

2.1 Introduction

The term "germination" is normally used to cover those processes which begin with the uptake of water by dry seed and successfully terminate with the emergence of redicle/ hypocotyl through the coverings of the seed. These observable morphological changes are a cumulative effect of cell division, cell enlargement and an increase in the metabolic activity of the embryo axis, endosperm and/or cotyledons.

The reserves in the germinating seeds are mobilized largely as a result of the activity of enzymes whose induction is under the control of "hormonal" factor released from the embryo and/or axes. The reason for this is that in the best understood system - the cereal grain - mobilization of food reserves is quite clearly under-hormonal control. However, there is no clear answer to the question of how food mobilization in non-cereal seeds is regulated. The main cause of this uncertainty is that when the trials are made to manipulate seeds, other than cereals, in the same manner. One cannot avoid producing damaged cotyledons and endosperm from which the testa often has had to be removed.

The review includes the sequence of events taking place in the process of germination and their regulation by the factors borned within the seeds or applied exogenously.

2.2 Hormones

Plant hormones are organic compounds, which are neither a source of energy nor act as building block, but synthesized in one part of the plant and translocated to another part, where in very low concentrations, induce a physiological response. In the target organ, this response need not be promotive, because process such as growth or differentiation are sometimes inhibited by hormones. Sucrose is not considered a hormone even though it is synthesized and translocated by plants, because it causes growth only at relatively high concentrations. Hormones are usually effective at internal concentrations of 1 µM or even less.

2.2.1 Auxins :

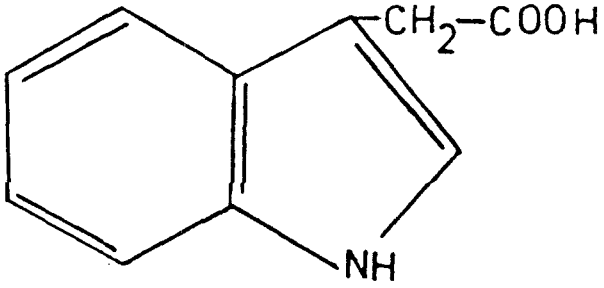
Frits Went in 1926 first used the term auxin for some unidentified compound which probably caused curvature in oat coleoptiles toward light. The coleoptile tip was reported to have abundant quantity of this compound as compared with other organs or tissues. It was later on identified as Indole-3-acetic acid (IAA). Plants contain two other compounds that cause many of the same responses as IAA, therefore, should be considered to be natural auxins

(Wightman and Lighty, 1982), one of these is 4-chloro-indole-acetic acid (4-chloro IAA) extracted from young seeds of various legumes (Engvild, 1986). Another, phenylacetic acid (PAA) is also of wide occurrence in plants. In addition to these, three additional compounds of natural occurrence namely - indole ethanol, indoleacetaldehyde and indoleaceto-nitrile have considerable auxin activity.

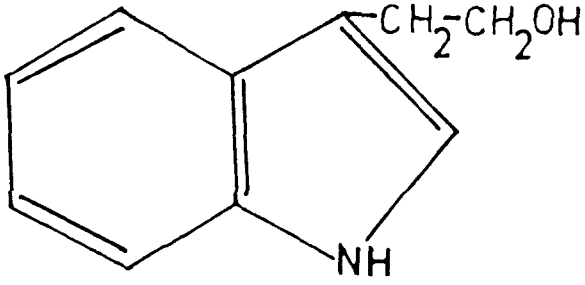
Some of the synthetic compounds are also classified as auxins because many of the physiological responses induced by them in the plants are similar to that of IAA, but, they are named as plant growth regulators. The best known out of them are naphthalene acetic acid (NAA), indole-3-butryric acid (IBA), 2,4-dichlorophenoxy acetic acid (2,4-D), 2,4,5-tri-chlorophenoxy acetic acid (2,4,5-T) and 2-methyl-4-chloro-phenoxyacetic acid (MCPA).

2.2.2 Gibberellins :

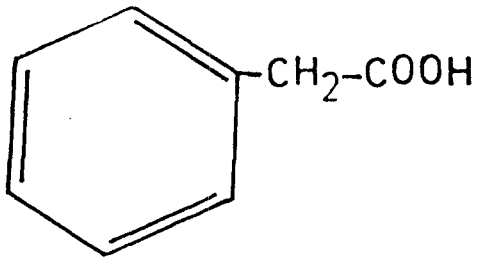
Yabuta and Hayashi in 1935 for the first time isolated this active substance from Gibberella fujikuroi which was later on named as gibberellin. Since then eighty four gibberellins (Salisbury and Ross, 1992) have been extracted from different lower and higher plants. However, most of the species have only few and at the most 15 (Phinney, 1979; Jones and MacMillan, 1984).



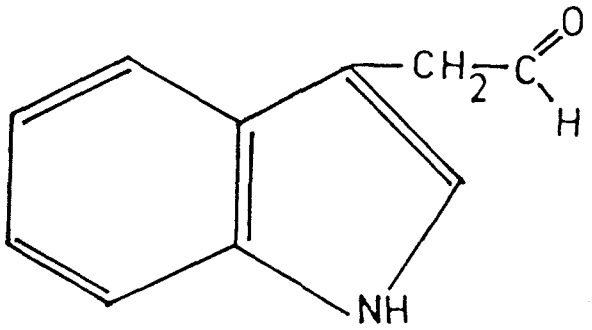
Indole acetic acid (IAA)



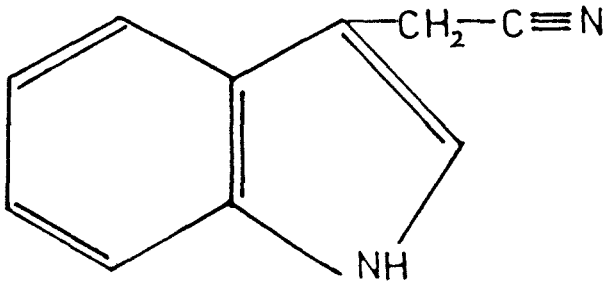
Indole Ethanol



Phenyl acetic acid

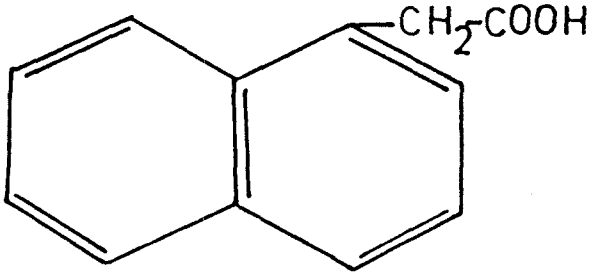


Indole acetaldehyde

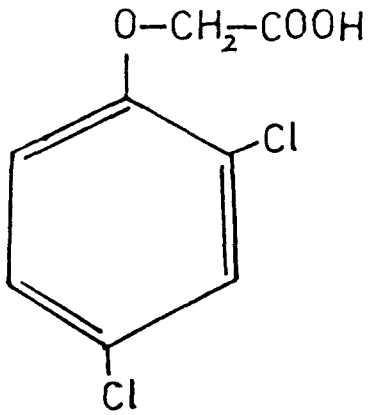


Indoleacetonitrile

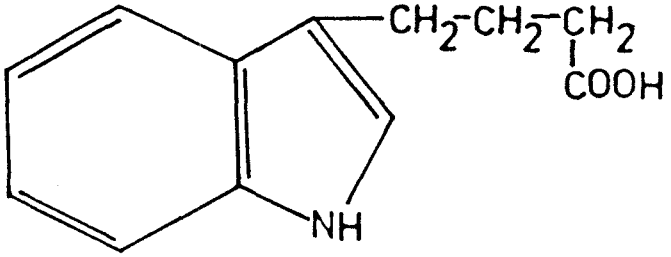
Naturally occurring auxins and auxin precursors



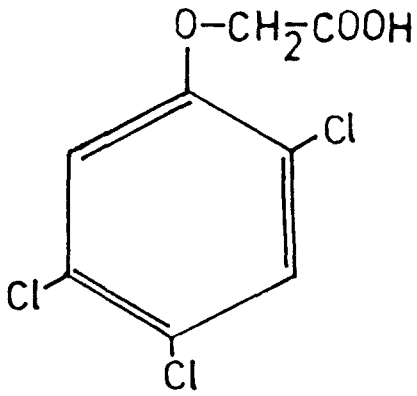
α naphthalene acetic acid (NAA)



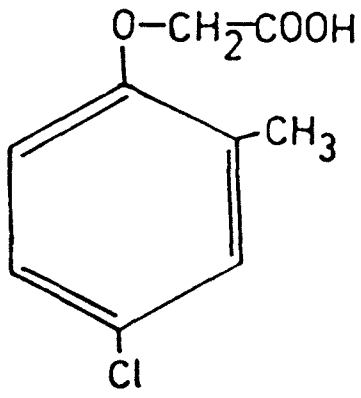
2,4-D



Indole butyric acid (IBA)

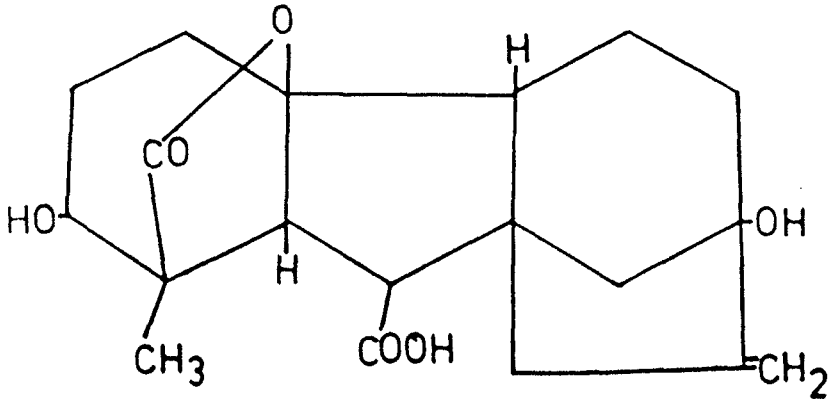


2,4,5-T

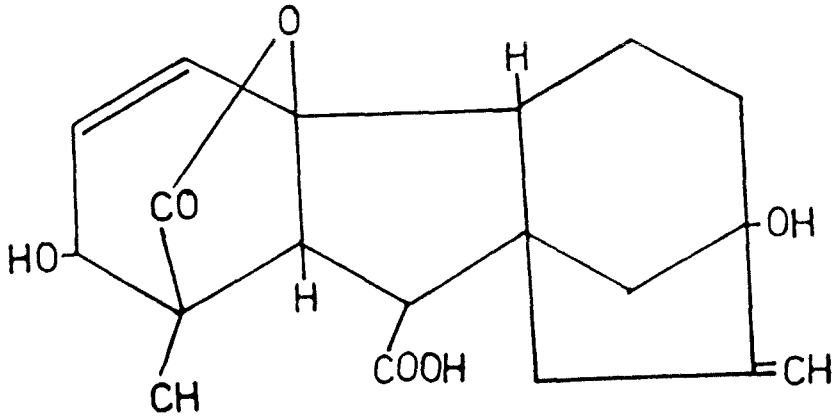


MCPA

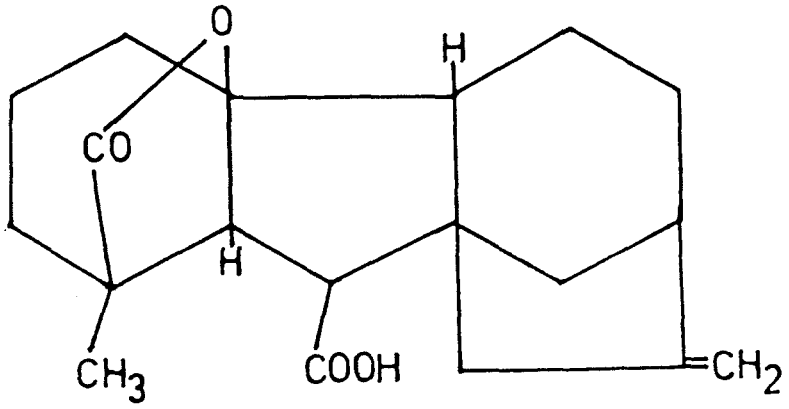
Synthetic auxins



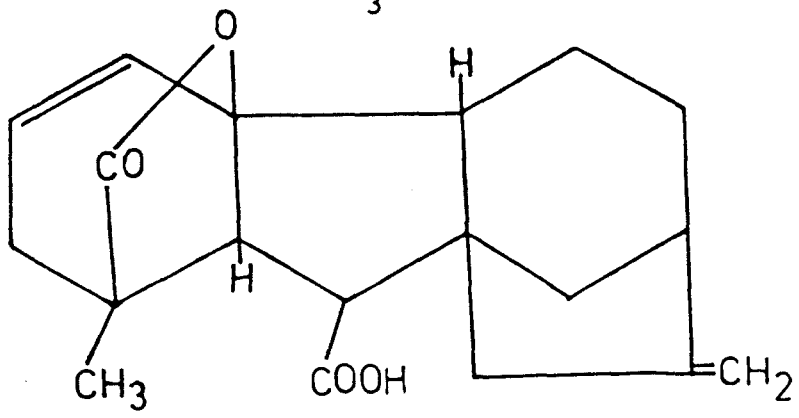
GA_1



GA_3



GA_4



GA_7

Structures of four highly active gibberillins

2.2.3 Cytokinins :

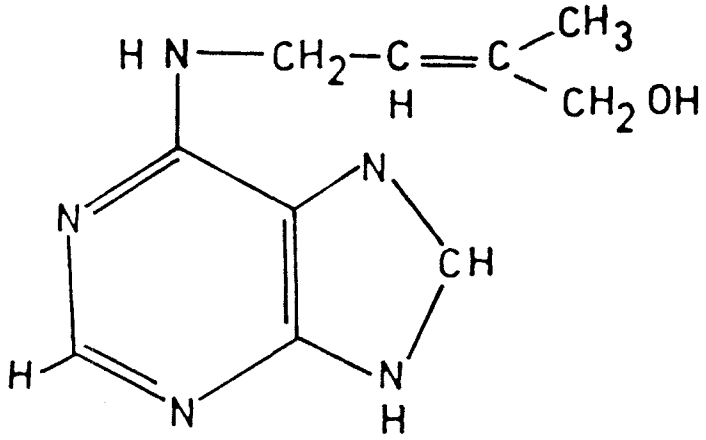
The vascular tissues in a plant system were proposed to be a rich source of this cell division factor (G.Haberlandt, 1913). Liquid endosperm of coconut was found to be another natural source inducing cytokinesis in the cells (J.V.Overbeek, 1941). However, the active compound was identified from the breakdown products of aged or autoclaved herring sperm DNA and was named as kinetin (Miller et al. 1956).

2.2.4 Ethylene :

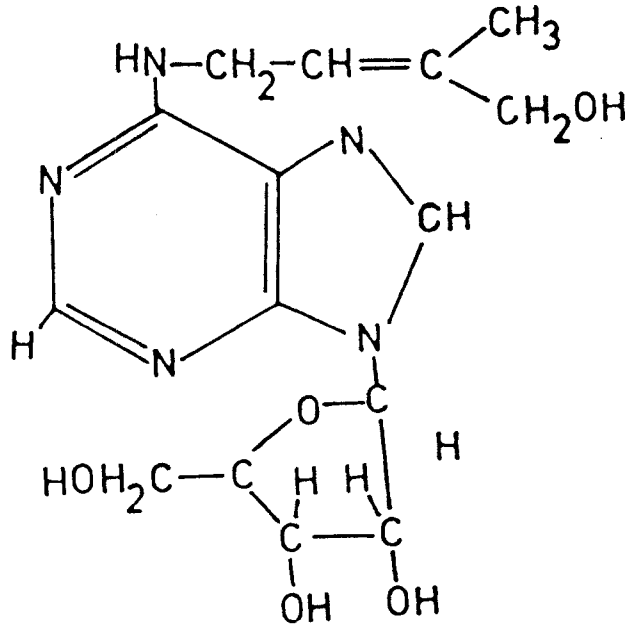
Ethylene is a unique plant hormone because of its structural simplicity and gaseous nature. Its involvement as a plant hormone was first noticed by Neljubow in 1901 as a triple response in etiolated pea seedlings.

2.2.5 Abscissic Acid :

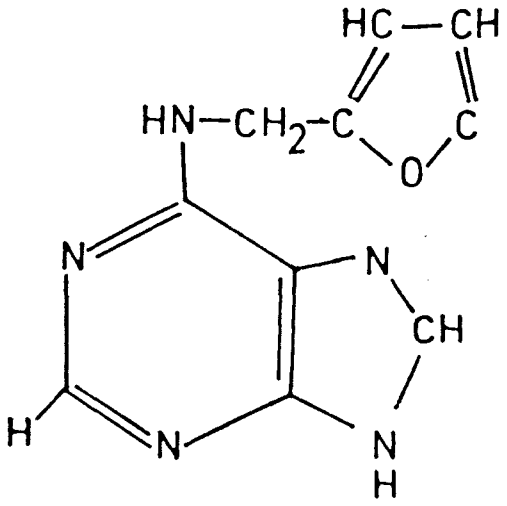
Liu and Carns (1961) isolated abscisin-I from mature cotton fruits, which stimulated abscision of debladed cotton petioles. Similarly, another substance, abscisin-II, was extracted from young cotton fruits by Ohkuma and associates (1963); Eagles and Wareing (1963) characterized "dormin" which was extracted from birch leaves held under short-day conditions. This substance when applied to the leaves of birch seedlings completely arrested its apical growth. In the year 1965, all the above substances (abscisin I, abscisin II and dormin) were found to be identical, therefore, termed as abscissic acid (ABA).



Zeatin



Zeatin riboside or ribosyl Zeatin



Kinetin

Structures of cytokinins

2.3 Metabolic changes associated with seed germination and growth of seedlings

Although, the exact sequences of events in seed germination varies among different plant species, the basic processes are similar. It includes imbibition, cell expansion, hydrolysis of food reserves in endosperm or cotyledons, transport of soluble metabolites to the embryo, and synthesis of cellular constituents in the embryo accompanied by cell division.

Morohashi and Shimokoriyama (1974) autoradiographed the cotyledons of Phaseolus mungo and Pisum sativum fed with glucose ^{14}C or indoleacetic acid ^{14}C and observed that these chemicals were confined and metabolised in the peripheral region of the cotyledons, during the early period of germination. This led them to conclude that in the storage parenchyma of the mature cotyledons the physiological activity was not uniformly distributed but remained localized in the peripheral region, at the initial stages of germination.

Knypl and Krystyna (1979) reported that the induced synthesis of nitrate reductase required the presence of light in intact cotyledons of 2-day old seedlings of Lactuca sativa. The efficiency was enhanced by the addition of molybdenum. However benzyladenine, gibberellic acid, succinic acid and 2,2-dimethylhydroxide reduced the activity of the enzyme. Trimethyl ammonium chloride did not affect NRA but markedly

inhibited greening and protein synthesis. KNO_3 on the other hand, stimulated protein synthesis and growth of cotyledons.

Maria and Antonio (1981) determined the pattern of *in vivo* activity of nitrate reductase (NR) in chlorophyllous cotyledons of soybean seedlings, cultivated at constant temperature and continuous light. They noted that chlorophyll accumulation preceded and approximately paralleled NRA indicating that nitrate reduction was associated with the development of anabolic processes in the cotyledons. The enzyme activity was inhibited by cycloheximide if added at the beginning of the illumination period, however the effect disappeared as the tissues were treated at later stages after being exposed to light. Another inhibitor actinomycin-D did not effect enzyme activity. This led them to conclude that cotyledons of germinating seeds possessed preformed mRNA for nitrate reductase, but depended, in part, on *de novo* protein synthesis.

Davies *et al.* (1981) observed that in cucumber cotyledons white light provided either continuously or as short, daily exposures stimulated the degradation process of neutral lipids and the activity of lipase. Higher levels of the enzyme activity developed in continuous white light. They have concluded that the influence of light or lipid degradation and related enzyme activities was prominent in the potentially photosynthetic cotyledons, therefore, species

dependent. However, the treatment had little effect on either the rate of development or the activities of glyoxylate cycle enzymes.

Martin and Northcote (1982) noted an increase in the water content of castor bean seeds and this brought about an increase in their fresh weight quicker by gibberellic acid treatment than the water soaked control. GA_3 also stimulated lipid metabolism by increasing the activities of most associated enzymes. What appeared to them was that GA_3, instead of stimulating particular enzymes, affected protein synthesis through the advanced appearence of larger quantities of total RNA (rRNA) and poly (A^+) RNA.

Melcher (1983) reported that the cotyledons of Pisum sativum seedlings grown in the presence or absence of light possessed a similar rate of breakdown of reserve proteins and the transport of breakdown products from the cotyledons. However, different parts of the seedling received varied quantities of nitrogen containing compounds. From day 4 of germination onwards, seedlings growing in light possessed most of the nitrogen containing compounds in the root whereas those grown in the dark had most of it in the shoot. The free amino acid, particularly that of homoserine and asparagine, composition of light or dark grown seedlings showed differences.

Giunashvili (1983) irradiated etiolated cotyledons of Cucumis sativus with red light pulses (650 nm, 5 min) and observed no change in the basal level of NRA. However, nitrate induced activity of the enzyme was stimulated by the treatment in the early stages of induction depending on the number and intervals between pulses. It was also suggested that the phytochrome stimulated NRA in earlier stages of its induction.

In germinating seeds, the activation of α-amylase, in the endosperm is known to occur because of the co-ordinated interaction between the embryo and aleurone layer cells. The germinating embryo is known to produce hormones which prompted Arstruni and Panosyan (1984) to incorporate in their studies the effect of β-IAA and kinetin with GA on the enzyme activity during germination of old and fresh mature wheat seeds. They observed that after 72 hours both in the endosperm and aleurone layer cells, in seeds subjected to storage enzyme activity increased 3.5 fold where as in young mature seeds the increase was 7-fold. The use of GA, β-IAA and kinetin led them to conclude that low intensity of the process of α-amylase, activation in the endosperm and aleurone layers of chronologically old seeds at early stages of germination was determined not by functional inadequacy of aleurone layer cell, producer of hydrolase enzymes, but due to the suppression of processes responsible for manufacture and co-ordinated dispatch to the endosperm aleurone

layer of β-IAA and kinetin which (together with GA) take part in regulating α-amylase activity of the endosperm of germinating seeds.

Ilahi (1985) reported higher concentrations of growth inhibiting substances than growth promoting substances in the germinating seeds of Pinus roxburgii and Pinus wallichiana stratified for 15 and 21 days respectively. However, an extension of the period of stratification decreased the level of inhibitors and increased that of growth promoting substances. He was able to identify four growth promoting and one inhibitory substances in germinating seeds.

Massanori (1986) while analysing the effect of red, far-red and dark on the total and basic peroxidases in the germinating seeds of Cucumis anguria reported an enhancement of basic peroxidases both by red and far-red light.

Nakagawa et al. (1986) results showed that NRs from spinach leaves and roots of different ages appeared to be identical proteins and this NADH-nitrate reductase (NR) activity per g fresh weight decreased as the seedling aged. However, they also reported that the NR molecule remains essentially the same throughout the ageing process.

Srivastava and Mungre (1986) reported an inhibition in the induction of nitrate reductase in excised cotyledons of radish seedling by their treatment with polyamines.

spermidine and spermine both in the presence or absence of light. However, putrescine had no effect. They also worked out a possible mechanism by which NR is inhibited by spermine. It neither affects the uptake of nitrate nor the breakdown of NR but inhibits de novo synthesis of NR.

Yandow and Richard (1986) observed an induction of NR by nitrate in the primary roots of Picea rubens but depressed by ammonia (NH_3). The seedlings were provided with nutrient solution of pH 3,4 or 5 and supplemented with salts of Al, Cd, B or Zn each at two concentrations. NR induction was found to be maximum at pH 3 upto day 42 whereas at pH 4 and 5 it was low. The presence of metal ions at pH 4 favoured induction of NR, however, suppressed it at pH 3 and 5.

Brown et al. (1986) used isolated chloroplast suspension and cell free system from light grown Pisum sativum to demonstrate the pathway of IAA catabolism. Decarboxylation oxidation was found to be the major process responsible for the destruction of IAA. The rate of IAA catabolism was higher in the presence of light than in its absence. They concluded that exogenous IAA was enzymetically decarboxylated by pea chloroplast fractions where indole-3-methanol (IM) was the important catabolite of light enhanced process.

Okuda et al. (1987) studied the changes in the level of some enzymes in the embryonic axes of germinating soybean (Glycine max) seeds, 48 hours after germination to

correlate it with the gene expression. Acid phosphatase level increased 27 times (at 40 hours), over those from dry seeds, due to de novo synthesis of enzyme protein in the embryo axes. Another enzyme, L-malate dehydrogenase increased 80 times (at 48 hours). However, it was mainly due to the activation of already existing enzyme protein in the axes by imbibition rather than being transferred from any where else or de novo synthesis of the enzyme protein.

Zhange et al. (1987) noted an increase in the inducibility of NR in the callus tissues of tobacco pre-treated with cytokinin for 12 hours in the presence of nitrate. They proposed that probably there are two major steps in the synthesis of nitrate reductase : (i) the synthesis of inactive NR apoenzyme which is stimulated by the presence of cytokinin and (ii) the activation of the NR apoenzyme, which requires the mediation of nitrate.

Sandberg and Ernstsen (1987) reported a rise in the free IAA contents of the seeds of Picea abies from 20 mg g^{-1} to 60 mg g^{-1} (dry weight) in the first five days of germination, thereafter, it decreased to 20 mg g^{-1}. However, the auxin released on alkaline hydrolysis decreased from its original level of 110 mg g^{-1} to 5-10 mg g^{-1} during the first week of germination.

Chiatante et al. (1988) observed an inhibition of chlorophyll accumulation in the cotyledons of lettuce

seedlings treated with cytokinin. It seemed to them to be independent from the type of modification in N_6 or N_9 position of the purine ring of the cytokinin. Therefore, they proposed that this particular behaviour might have been induced by the purine ring of the hormone for which no specific mechanism of action was suggested by them.

Deshmukh et al. (1988) reported a positive effect of GA_3 on the α-amylase activity in the endosperm of germinating barley seeds, however, ABA inhibited it. So they proposed that the level of GA_3 and ABA regulate the activity of the enzyme through an inhibitor protein. It was self evident from their results that the amount of this inhibitor protein decreased in the presence of GA_3 but increased in the presence of ABA in both aleurone layer and medium at 20 and 24 hours of the incubation. The use of GA_3 and ABA together made the protein band to be less prominent as compared with that in ABA alone.

Bashist (1988) in the seedlings of Sesamum indicum treated with gibberellic acid and grown in a standard nutrient medium with continuous illumination noted poor nitrate uptake and its reduction. However, the ill effect induced by the treatment was overcome by the addition of sucrose (2%) to the medium. He, therefore, proposed that the overall effect of the GA was modified by the lower sugar level of the tissue but could be regularised by its exogenous application.

Dencheva and Klisurska (1988) observed that the growth inhibitor CCC retarded the germination and growth of the seedlings of maize. This was associated with an increase in the quantity of phenols, total peroxidases and IAA oxidase. The enhanced level of the respective isoenzymes was found to be main reason for the increased level of peroxidases and IAA oxidases.They, therefore, proposed that CCC affects the plant system in different ways and proposed a model to explain the possible mechanism by which the inhibitor operates its effect.

Sharma (1988) noted that neither GA nor casein-hydrolysate added exogenously to the germinating seeds of chickpea-could affect the protease development in the cotyledons. However, the presence of intact embryo axis favoured the mobilization of reserve proteins through the production of proteolytic enzymes in the cotyledons.

Basra et al. (1989) observed that the treatment of maize seeds with phthalimide AC 94377 (1-(3-chlorophthalimide) cyclohexane carboxyamide), GA_{4+7} and ABA significantly increased germinability under sub and supra optimal temperature regimes. ABA was specially stimulatory at sub-optimal temperature. Metabolic changes revealed an increased accumulation of soluble sugars and protein as compared with the controls under-stressing temperature. The activities of acid phosphatase, invertase, catalase

and peroxidase were seemingly related with the altered metabolism. They, therefore, proposed that the level gibberellic acid and abscissic acid regulate the system for stress allevation.

Tahir and Farooq (1989) assessed seedlings, leaves and the developing grains for *in vivo* nitrate reductase activity (NRA) of four species of *Fagopyrum* (viz. *F. esculentum* Moench, *F. saqittatum*, *F. tatarium* and *F. kashmirianum*). In all the four species, the leaves exhibited a decreasing trend in NRA with the advancement of their age and it was positively correlated with the nitrate level of the tissue. However, in grains the NRA level increased with each successive stage of its development. Among the various species, *F. tatarium* maintained higher NR activity in the grains during their development.

Gilmanov and Sultanbaey (1989) germinated the grains of *Triticum durum* pre-treated with 6-BAP, 2,4-D, kinetin, IAA or GA and analysed them for NADP-glutamate dehydrogenase (NADP-GDH). 2,4-D induced the formation of the enzyme in the non-embryonic part of the grain. They proposed that primary cytokinins act on the embryonic fragments which was closely followed by the action of a hypothetical compound in the non-embryonic part of the grain, similar in action to that of 2,4-D, inducing the formation of NADP-GDH.

Karimov and Dontsova (1989) reported that exogenously supplied abscissic acid (ABA) to germinating seeds of cotton inhibited the synthesis of various RNA-fractions and the formation of ribosomes and polysomes. The presence of the hormone induced polysome degradation which led to an increase in the level of ribosomes not involved in protein synthesis. This also resulted changes in the correlation of synthesized RNA molecules and ribonucleoprotein particles in cotton seedlings.

Karunagaran and Rao (1990) reported that the activity of protease was nine fold in the attached cotyledons and proteins declined faster than in the detached cotyledons where the level of enzyme was three fold from 0 to day 3. After day 2, the amino acids did not accumulate in the attached cotyledons, contrarily they accumulated continuously in the detached cotyledons. This led them to conclude that in the cotyledons of germinating seeds of Macrotyloma uniflorum, the activity of protease, protein degradation and the utilization of amino acids was dependent on the presence of the embryonic axes. This de novo synthesis of the enzyme regulated by the axes was not replaceable by exogenous addition of either gibberellic acid or benzyladenine. They further suggested that the embryonic axes acted as a sink, during the germination of the seed, for the hydrolytic products and its absence lead to the accumulation of these end products restricting further enzyme

activity. Similarly, in another observation, Karunagaran et al. (1992) found the dependence of the activity of amylase and protease and the degradation of starch and protein in the cotyledons of germinating seeds of cowpea on the presence of embryo axes.

Saha (1992) observed the presence of cytokinin activity in germinating pea seeds only after 24 hours. The bound cytokinin (zeatin glucoside) decreased after 48 hours but the free cytokinin (zeatin and zeatin riboside) increased. It was suggested that a control mechanism existed where a definite amount of bound cytokinin were hydrolysed to free bases. Cytokinin was said to control protein turn over and transport of gibberellins from the embryo to induce other biochemical processes in germinating seeds.

2.4 Effect of chemicals on the germination of seeds

All of the active substances (auxins, gibberellins, cytokinins, abscissic acid and ethylene) are of natural occurrence in the seeds. However, numerous species of seed still respond to these substances, supplied exogenously either singly or in combination. Therefore, some of these chemicals have a commercial value in agriculture for stimulating the rate of seed germination.

Jacobson (1978) treated pea seeds with 2,4,-D (a synthetic auxin), (a) at the beginning of the imbibition

and (b) after 12 hours of imbibition in water. Auxin application reduced the fresh weight increase but enhanced dry weight decrease after 12, 72 and 144 hours of germination in the dark. The activity of acidic cytoplasmic RNase in the 30,000 xg supernatant of the homogenate, 2,4-D caused no significant effect from 12 to 72 hours but from 72 to 144 hours, the enzyme activity was lower than in the control. However, in the 30,000 xg supernatants of the dialyzed and concentrated homogenate the activity was stimulated from 12 to 72 hours and exhibited a parallel decrease from 72 to 144 hours in control and both the hormone treatments.

Thomas (1984) observed that the dried pre-soaked seeds of celery in the solution of GA_4 or GA_7 plus ethaphon (G + E) or an osmotic primary treatment in light with polyethylene glycol (PEG) were less thermoinhibited. The seeds treated with G + E germinated after a week where as PEG treated seeds germinated after 3 days at 18 and 25°C, in the dark. However, untreated seeds did not germinate. Irrespective of the treatment, dry seeds exhibited very little cytokinin activity. However, the control seeds imbibed for 18 hours in dark, possessed higher cytokinin activity than both G + E and PEG treated seeds.

Setia and Narang (1985a) increased the level of salinity by supplementing the medium with NaCl (50, 100 and 200 mM) and observed that the per cent germination

declined progressively with an increase in the concentration of the salt. The length, fresh and dry weight of the resulting seedlings also decreased. Three growth regulators (GA_3, kinetin, morphactin) were also used at a concentration of 5 mg/ml each, to counteract the effect of salinity but all of them had an additive effect of salinity on seed germination. Similarly in other study Setia and Narang (1985b) reported an interaction effect of kinetin, IAA and GA_3 (1 and 10 µg/ml) with the salinity induced by $NaHCO_3$ (50, 100 and 150 mM) on the germination of pea seeds. Salinity decreased the germination percentage to 35%, however, it improved with the addition of either of the above hormones. GA was more effective than kinetin and IAA enhanced germination to a level comparable with the control. They, therefore, suggested the use of these plant hormones to overcome the inhibition of seed germination in saline conditions.

Sisler and Carmen (1986) exposed tobacco (Nicotiana tabaccum) seeds to ethylene during their germination, they noted slight improvement by the treatment. The germination was, however, completely inhibited if insted of ethylene its inhibitors (2,5-norbornadiene aminoxyacetic acid and $AgNO_3$) were used. This inhibition by these chemicals can partly be overcome by ethylene. This led them to conclude that tobacco seeds require a certain level of ethylene

for their germination which was supplied by its own metabolism.

Kepczynski (1986) noted an inhibition in the germination of Amaranthus caudatus seeds treated with tetcyclasis (BAS 106), an inhibitor of GA biosynthesis. The effect of BAS 106 was antagonised by gibberellin (GA_{4+7}). The ethylene released from ethephon (2-chloroethyl phosphoric acid) or synthesized from ACC (1-amino cyclopropane-1-carboxylic acid) mimicked the effects of gibberellin. However, seed germination could not be stimulated by gibberellin in the presence of amino ethoxy vinylglycine (AVG), an inhibitor of ethylene biosynthesis; Gibberellin did not reverse the effect of BAS 106 if AVG was added simultaneously. These observations were further confirmed in their later studies - Kepczynski and Kepczynska (1988) and Kepczynski et al (1988). Paclobutrazol a specific inhibitor of gibberellin synthesis which blocks the biosynthetic step from ent-kaurene to ent-kaurenoic acid was used to inhibit the seed germination of A. paniculatus. This inhibition was counteracted by gibberellin A_3 (GA_3), ethephon or ACC. It was worth to note that ethephon was more effective than GA when applied simultaneously with paclobutrozol. The seeds incubated with paclobutrazol for 5 days decreased sensitivity to GA_3 and ethephon. It was, therefore, concluded that endogenous gibberellin are required for the germination of these seeds. Such simulation action of endogenous

gibberellins by ethylene may indicate that ethylene and gibberellins, effect the same or similar processes leading to germination.

Bradow et al. (1988) reported that four synthetic multiring analogs of strigol, a naturally occuring sesquiterpene lactone that promotes germination of dormant seeds of striga, were found to stimulate germination of dormant Lactuca seeds. The effects on light sensitive and light insensitive lettuce seeds were concentration depend and exceed those produced by equimolar solution of gibberellins, strigol and epistrigol, promoted lettuce seed germination to a lesser degree than did the synthetic analogs.

Patel and Chatterji (1988) studied the effect of growth regulators on seed germination and growth of seedling of papaya. The seeds soaked in NAA, IAA, IBA, β -NPA and GA for 24 hours resulted in better germination and quicker growth of seedlings. They recommended that the papaya seeds must be soaked in the solution of IAA (100 ppm) for better germination and that of GA (100 ppm) for better growth of seedling prior to their sowing.

Pressman and Shaked (1988) observed that the seeds of Apium graveolens collected from the plants pre-treated with paclobutrazol at the time of anthesis had a marked reduction in germination in light and failed to germinate in the dark. This inhibitory effect was overcome by the

addition of $GA_{4/7}$ to the imbibition solution but GA_3 was ineffective. It led them to suggest that pacloburtrazols, application to mother plant inhibited biosynthesis of GAs, which normally enable the germination of annual seeds under unfavourable conditions. However, the exogenous application of $GA_{4/7}$ fulfills the need.

Grubisic et al. (1988) reported that the seeds of Paulownia tomentosa may be subjected to germinate if exposed to red light or soaked in the solution of GA. The effect of red light was completely neutralized by far red light, abscissic acid or some growth retardants (ancymidol, tetcyclasis and paclobutrazol) but not by AMO 1618 or chlorocholine chloride. The inhibition induced by far-red light and some growth retardants was overcome by gibberellins. The effect of abscisic acid was reversed by fusicoccin, which did not reverse the inhibition caused by far-red light or growth retardants. They further noted that the germination of light insensitive wheat, corn, alfalfa and mungbean seeds was not inhibited by growth retardants.

2.5 Loss of solutes during germination

The seeds, at the initial stages of imbibition, from the outermost layers of cells lose solutes (sugars, proteins, amino acids and electrolytes) into the surrounding medium. However, the rate of loss decreases with an increase in the

degree of hydration of the tissues. This phenomenon is purely passive (Simon and Harun, 1974) and is due to the fact that phospholipids with a water content of less than 20% are claimed to adopt a hexagonal configuration so, the membrane in this state is not lameller but possess pores (Luzzati and Husson, 1962). Therefore solutes leak through them until the lamellar bilayer is established when normal physiological control system would become operative (Wilkins, 1987).

Simon and Harun (1972) noted that solutes leaked from the seeds of Ricinus and pea embryos at a rate which declined rapidly at first and then more slowly. Embryos pre-dried over calcium chloride and then hydrated, followed the same leakage pattern. However, pea embryos if allowed to imbibe some water through a small part of their surface, leak relatively slowly when subsequently immersed in water. Here they also reported that the greater the initial imbibition the slower the subsequent leakage. They finally suggested that during the course of seed drying the membrane looses its structure. However, this integrity was re-established when the seeds were allowed to imbibe water for a short period.

Simon and Wiebe (1975) observed that the water content of the seed determines the degree of leakage of electrolytes from pea embryos in the initial stages of

imbibition. The loss was very slow if the embryos had a water content of 30% or more (water potential higher than 80 bars). Seeds that have reached this degree hydration are killed by a 20 minute exposure to liquid nitrogen.

Hendrick and Taylorson (1976) imbibed the seeds of Avena fatua, Lactuca sativa, Barbarea vulgaris, Amaranthus albus, Setaria faberi and Setaria viridis to study the leakages of amino acids and/or fluorescent material at temperatures ranging from 15 to 40°C. They found an enhanced rate of leakage of the amino acids from 8 of the 10 kinds of seeds studied which indicated prominent increase in permeability of the plasmalemma. The seeds of A. albus and A. theophrasti did not show membrane change in the 25 to 40°C range, therefore germinated best at 35° to 40°C. The seeds of the rest of the cultivars germinated best at a temperature below 30°C.

Mathews and Rogerson (1976) reported that differences in the leaching of solutes from different seed lots of peas were associated with the condition of the embryo and not the tastae. Seeds with areas of dead tissue on the abaxial surface of their cotyledons exhibited the highest levels of leaching but the large range of levels found among seed lots resulted from differences in leaching from living tissues. A rapid decline in the leaching of solutes was noted from the first minute in water and was related to

the ability of the embryo to retain solutes rather than their solute content when dry embryos were placed in water.

Samad and Pearce (1978) noted leakage of reducing sugars, phosphates, potassium, total electrically conducting materials, proteins and phenolics from the seeds of two varieties of Arachis hypogea in the first 24 hours of imbibition in distilled water. The seeds without testa leached larger quantities of all of the substances, except protein and phenolics, than those with intact testa. Sugar, phosphates, potassium ions and total electrically conducting material leached at fastest rate in first hours of imbibition. The presence of peroxidase and catalase was noticed only in the leachates of the seeds with testa. This led them to conclude that the testa of peanut was not a barrier to uptake of water and that leaching of some substances was greater in its absence than in its presence. They did not agree with the suggestion of others that the greater leaching and reduced viability and vigour of seeds whose testa has been removed, was due to more rapid imbibition in the absence of testa. However, they proposed that the phenolics (leachable in water) in the testa of peanut seeds acted as antioxidants which supplemented the protection afforded by α -tocopherol (which is lipid soluble) to cell components against oxidation.

Leopold (1980) studied the effect of low temperature on the rates of water uptake into and solute leakage out of

cotyledons of soybean. Arrhenius plots of water entry show no significant difference into living than into heat killed tissue which supported the concept of slight hinderance by membrane in the passage of water through the tissues. However, Arrhenius plots of solute leakage in living tissue exhibited 10-fold lower rate than the dead tissues. This led them to conclude that at low temperature (15^{o}C) membranes may be reorganised in a faulty manner and show lessened effectiveness as a barrier.

McKersie and Stinson (1980) germinated the seeds of Lotus corniculatus cv. carroll for 0, 12 and 24 hours and then subjected to dehydration to study the damage in the membrane. The desiccation tolerant (0 and 12 hours) seeds, during reimbibition, leaked lesser quantities of all solutes (i.e. total electrolytes, potassium, phosphate, sugar, amino acids and protein). Desiccation sensitive (24 hours) seeds higher levels, but evidence of selective permeability remained. It was suggested that the plasmalemma was not ruptured or torn by the dehydration treatment, but a more subtle structural alterations occurred. X-ray diffraction revealed the possibility of the formation of a hexagonal rather than a lamellar phase in seed membranes, at moisture contents below 20%. Phospholipids were extracted from desiccation tolerant (0 hours) and desiccation sensitive (24 hours) seeds and hydrated to 5,10,20 and 40% water. This

phospholipid water system was found to be lamellar even at 5% water. They finally concluded that the membrane damage and the leakage of cytoplasmic solutes from seed could not be explained by the formation of a hexagonal phase by membrane phospholipids.

Becwar et al. (1982) examined the effect of dehydration on the leakage of electrolytes and viability in two species of seeds that do not servive desiccation. Leakage from Acer saccharinum seeds increased markedly as seed moisture content fell from 45 to 35% and germination decreased from 97 to 5%. The embryos of Chrysalidocarpus lutescens showed an increase in both initial leakage and steady state leakage rates in response to dehydration from an original moisture content of 84 to as low as 53%. It was suggested that the membrane in the desiccation sensitive seed tissue were damaged by dehydration below a critical moisture content, 40% in A. saccharinum seeds and 55% in C. lutescens embryos. This damage of the membrane contributes dehydration induced loss of viability.

Murphy and Noland (1982) evaluated the possibility of the involvement of membrane and water viscosity in the temperature effects on imbibition and solute leakage by radish seeds and excised sugar pine embryos. The initial rates of water uptake and leakage of solutes increased as the temperature was raised through the range from 5 to 35^{o}C.

It was accounted for, primarily by changes in the viscosity of the imbibition medium. Heat killed seeds and embryos had significantly higher rates of imbibition and leakage of solute than did viable ones. It clearly indicated the involvement of the membrane in both these processes.

Givelberg et al. (1984) reported a higher rate of leakage of electrolytes from the seeds of Solanum nigrum with an increase in temperature. The increase was prominent in the first 3 hours of imbibition. The leakage of sodium ion was almost complete after 6 hours of imbibition both at 25 and 50°C but much more leaked at higher temperature. A marked increase in the rate of leakage of potassium occurred after 24 hours at 50°C, therefore, after 96 hours three times more potassium was lost at this temperature than at 25°C. Calcium and potassium continued to leak even after 6 hours with the same rate at both the temperatures. Higher temperature had a marked positive effect on the leakage of both proteins and nucleic acids. Malate dehydrogenase was not detected in the lechates at the initial stages of imbibition but it appeared after 48 hours at 50°C which proposed to be result of damage to the membrane.

Schoettle and Leopold (1984) reported that solute leakage from imbibed soybean seeds increased with accelerated agening in a linear manner in the range over which vigour was depressed. It was noteworthy that increase in

leakage with accelerated ageing were closely associated with increase in areas containing damaged cells as evident from staining with Evans-blue.

Doijode (1988) observed a decrease in the per cent germination of the seeds of eleven cultivars of Capsicum annuum with an increase in the period of induced ageing (40°C, 95% RH). The aged seeds exhibited higher electrical conductivity and the content of soluble sugar in the lechate. There was significant variation among the cultivars as regards the quantum of lechates, however it was more in those having poor germination percentage.

Doijode (1990) exposed the seeds of ten cultivars of Allium cepa to a temperature of 42°C and relative humidity of 95% for 1,2,4 and 8 days to induce artificial ageing. Out of ten only five cultivars (Arka Pragati, Poona Red, Chickballapur Local, Crystal Wax and Nasik Red Globe) had high percentage of germination after 4 days of ageing limit, none of the seeds germinated after 8 days of ageing. The process of ageing favoured the leaching of electrolytes and soluble sugars. The ageing period was positively correlated with the leaching of solutes and negatively with seed viability and vigour in various cultivars of onion.

2.6 Effect of chemicals on growth of seedlings

The growth of the seedlings largely depends on certain environmental factors which mainly includes light,

temperature and the availability of water and nutrients. However, equally important is the endogenous level of hormones, their interactions with themselves and also with the changing sensitivity of the tissues with growth. In addition to the natural hormones, there are many man made substances, very much similar in action to natural hormones, capable to modify the habits of seed germination and the growth of resulting seedlings, if added exogenously. These synthetic compounds are therefore, being used very extensively in treating the seeds and the seedlings to possibly under stand the mechanism of action of endogenous hormones and to improve plant productivity.

Pinfield et al. (1984) reported that exogenous application of 2,4-D (2-chloro-diphenoxy acetic acid) induced swelling of the hypocotyl base in the seedlings of marrow. This effect of the auxin was counteracted by presence of GA_3 or kinetin in the incubation medium. The inhibition induced by 2,4-D on the extension growth in the hypocotyl could also be overcome by GA_3. Another hormone ethaphone (ethylene releasing substance), induced responses very much similar to those of 2,4-D. However, most of these abnormalities disappeared if 2,4-D was associated with $CoCl_2$ in the medium. These observations lead them to conclude that the possible mechanism of the operation of 2,4-D in the induction of the above symptoms was through the

induced synthesis of ethylene in the tissue for which this auxin in very well recognised. At the same time auxin also inhibited the accumulation of endogenous gibberellins but enhanced the level of cytokinins during the development of seedlings.

Zhao-Zhou and Rui-chi (1984) noted an inhibition in the rate of elongation and a decrease in the fresh weight in etiolated seedlings of Pisum sativum exposed to ethylene. The effect was however, neutralized if ethylene and fusicoccin (FC) were supplied together. FC could not overcome the reduction of DNA synthesis and cell division, induced by ethylene. The cell wall microfibrils got deposited longitudinally under the influence of ethylene but their orientation changed to random deposition by the addition of FC. Fusicoccin also overcome the ill effect of ethylene on the uptake of K^+, H^+ secretion and the rate of respiration. They therefore, proposed that FC stimulated the rate of respiration, H^+ secretion and K^+ uptake, thus compelling the cell to take up more water to favour their swelling and elongation.

Nelson and Sharples (1986) pre-treated lettuce seeds with 0.5 mM of fusicoccin (FC) and incubated them at 33°C for 10 hours, alternating with 23°C. They observed an increase in the rate and total emergence of the seedlings which could be further improved if FC was associated with

GA or K. However, GA or K, if used alone, were not effective. The growth of the radicle was inhibited by FC, GA and/or K but both FC and GA stimulated the elongation of hypocotyl. The use of lower concentration (.05 mM) of FC exhibited the same total emergence as higher concentrations and the inhibitory effect on redicle growth was very much reduced but the rate of emergence was slower.

Grossmann et al. (1987) soaked the seeds of Glycine max in aqueous solutions of two growth retardants (tetcyclacis and LAB 150978) for 3 minutes. The seedlings raised from the seeds pre-treated with these chemicals, as compared with the control, were characterized with : (i) shoot growth was more markedly reduced than root growth. (ii) gibberellin content decreased, especially in the shoot tip (iii) the level of abscissic acid decreased, particularly in the primary leaf, epicotyl and the root. In contrast, the levels of trans-zeatin-riboside and dihydro-zeatin-riboside type cytokinins were elevated significantly mainly in the primary leaf, epicotyl and hypocotyl. However, the content of 3-indole acetic acid, in different parts changed only slightly. These observations compelled them to conclude that both these growth retardants not only decreased. The level of gibberellin but also interfered directly or indirectly with the regulation of the endogenous level of cytokinin and abscissic acid. This might explain the additional role of growth retardants in the treated plants e.g.

delayed senescence and enhanced chlorophyll concentration in the leaves.

Mathias et al. (1989) selected two cultivars of Pisum sativum, alaska and its dwarf mutant, deficient in endogenous gibberellins (progress), for studying the effect of exogenous GA on light induced greening of etiolated seedlings. Progress accumulated chlorophyll and light harvesting chlorophyll protein, associated with photosystem II, (LHC-II) more rapidly than alaska or GA treated seedlings of both the cultivars. Alaska seedlings had higher chlorophyll, after 24 hours, than their counterparts treated with GA. Both the cultivars (alaska and progress) when treated with GA exhibited identical pattern for LHC-II accumulation. They also had similar patterns of LHC-II mRNA induction before and after being treated with GA. It indicated that the differences in the induction of LHC-II mRNA were not responsible for the varied accumulation of LHC-II. In addition to this a significant correlation was found between chlorophyll and LHC-II content in each treatment. They, therefore, concluded that GA alters the progress of greening either directly or indirectly because it lead to the accumulation of chlorophyll and LHC-II in both the cultivars in the same way.

Rajput et al. (1990) observed that the autotrophic seedlings of Cascuta did not respond significantly in terms of shoot elongation, to the exogenous application of

gibberellin and abscissic acid both in the presence or absence of light. However, the seedlings treated with GA possessed low fresh weight and looked thinner than control. The other hormones, ethylene, cytokinin and higher concentration of auxin exhibited very marked growth inhibition which was accompanied by swelling of the stem. The seedlings treated with cytokinin exhibited differences in many respects from those treated with other hormones. The most prominent out of them was the formation of papillae. It was therefore, suggested that the autotrophic Cascuta seedlings respond to the hormones in some what the similar way except the formation of papillae and insensitivity to abscissic acid.

2.7 Concluding Remarks :

The available literature, reviewed above reveals that not much work has been done on growth hormones with regard to seed germination and leakage of solutes. Therefore, it is considered desirable to undertake detailed studies of the germination, leakage and growth of seedlings as influenced by growth hormones, temperature, light and dehydration.

CHAPTER - 3

MATERIALS AND METHODS

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MATERIALS AND METHODS

3.1 Proposed study

The following studies are proposed to be conducted with a goal to study the pattern of leakage of some of the substances from the seeds of two varieties of Pisum sativum (cv. Boneville and Arkel), into the soaking medium, during the early stages of imbibition, under varied conditions. In all, five experiments will be undertaken.

Experiment 1, is expected to indicate the native values of different components in the seeds of both the cultivars to be estimated, during these studies.

In experiment 2 to 4, the pattern of leakage of the substances and its relationship with the metabolic state of the cotyledons and embryo under varied conditions will be observed.

Lastly, in experiment 5, a regulatory role of certain exogenously applied hormone : on the leakage of the substances and seed metabolism, under similar conditions as in the previous experiments, will be approached.

3.2 Seeds

The seeds of two varieties of pea (namely Boneville and Arkel) will be obtained from National Seed Corporation,

I.A.R.I. New Delhi. The healthy seeds will be surface sterilized with 0.01% mercuric chloride and rinsed thrice with double distilled water.

3.3 Experiment 1

The dry seeds of both the varieties of pea will be powdered and chemically analysed for the following :

1. Nitrate contents
2. Soluble and insoluble carbohydrate contents
3. Soluble and insoluble protein contents
4. Ca^{++} and Na^{+} content, and
5. N P K contents

3.4 Experiment 2

The surface sterilized seeds of both the varieties will be allowed to germinate in separately plastic trays in sufficient quantity of ionized water at 24 $\pm$ 2°C in :

a) Dark
b) Diffused light (9 WM^{-2})
c) Bright light (18 WM^{-2})

The samples for chemical analysis will be picked at an interval of 3, 6, 12, 18, 24, 30, 36 and 48 hours, after soaking. At each sampling, moisture level of some of the seeds will be determined while in the rest the seed coat will be removed and the cotyledons and embryo will be analysed

separately for the following characteristics :

1. Nitrate reductase activity
2. Peroxidase activity
3. Nitrate contents
4. Soluble and insoluble carbohydrate contents
5. Soluble and insoluble protein contents
6. Ca^{++} and Na^{+} contents, and
7. N P K contents

3.5 Experiment 3

The surface sterilized seeds of both the varieties will be allowed to imbibe in petriplates with a known amount of double distilled water at 24 $\pm$ 2^{o}C in the

a) Dark
b) Diffused light (9 WM^{-2})
c) Bright light (18 WM^{-2})

These hydrated seeds with intact seed coat will be expected to leake certain substances into the soaking medium which will be analysed for the following, at an interval of 3,6,12,18,24,30,36 and 48 hours, after soaking in each set.

1. Nitrate contents
2. Total carbohydrate contents
3. Total protein contents
4. Ca^{++} and Na^{+} contents, and
5. N P K contents

3.6 Experiment 4

The surface sterilized seeds of both the varieties will be imbibed in ionized water for 1 or 2 hours in the presence or absence of light. These hydrated seeds will be dehydrated at 25, 30, 35 and 40^{o}C in light or dark. These, treated dehydrated seeds will again be hydrated in a known quantity of ionized water in :

a) Dark

b) Diffused light (9 WM^{-2})

c) Bright light (18 WM^{-2})

The soaking medium will be chemically analysed for the leaked substances as mentioned in experiment 3. Biological samples will be collected 3, 6, 12, 18, 24, 30, 36 and 48 hours, after rehydration. Some of the seeds, from each sampling, will be quantitatively analysed for their water content and the rest of them will be subjected to chemical analysis for the following characteristics in the cotyledons and embryo separately.

1. Nitrate reductase activity
2. Peroxidase activity
3. Nitrate contents
4. Soluble and insoluble carbohydrate contents
5. Soluble and insoluble protein contents
6. Ca^{++} and Na^{++} contents, and
7. N P K contents

3.7 Experiment 5

This final experiment will be performed in two phases :

(A) The surface sterilized seeds of both the varieties will be soaked in 10^{-10}, 10^{-8}, 10^{-6} and 10^{-4} M solutions of IAA, IBA and/or GA for 1 or 2 hours in either :

a) Dark

b) Diffused light (9 WM^{-2})

c) Bright light (18 WM^{-2})

The treated seeds will be washed with double distilled water to remove the adhering solution and then transferred to petriplates containing a known quantity of double distilled water. These plates will be placed in :

a) Dark

b) Diffused light (9 WM^{-2})

c) Bright light (18 WM^{-2})

In each set, the following substances in the soaking solution will be estimated at an interval of 3, 6, 12, 18, 24, 30, 36 and 48 hours after initial soaking period (it will also include the duration for which the seeds have been kept in the hormones solution).

1. Nitrate contents
2. Total carbohydrate contents
3. Total protein contents
4. Ca^{++} and Na^{+} contents, and

5. N P K contents

At each above sampling, the imbibed seeds left in petriplates, after the decantation of the soaking medium, will be subjected to chemical analysis. The seeds coat will be removed and the cotyledons and embryo will be analysed for the following components, separately

1. Nitrate reductase activity
2. Peroxidase activity
3. Nitrate contents
4. Soluble and insoluble carbohydrate contents
5. Soluble and insoluble protein contents
6. Ca^{++} and Na^{+} contents, and
7. N P K contents

(B) The surface sterilised seeds will be treated with the hormones in the same way as in A, above. These treated seeds from each set, will be dehydrated, separately in the presence or absence of light at 25, 30, 35 and 40^{o}C.

Treated, dehydrated seeds will then be allowed to imbibe in a known quantity of Double Distilled Water in petriplates separately under :

a) Dark
b) Diffused light (9 WM^{-2})
c) Bright light (18 WM^{-2})

The soaking medium and the biological samples will be assessed for different characteristics in the same way

as in A, above. Every sample, in all the experiments will be replicated, at least, three times. Each experiment will be repeated once.

3.8 Chemical analysis

The following parameters have been selected for chemical analysis in the biological material.

3.8.1 Estimation of Nitrate Reductase Activity :

Nitrate reductase activity (NRA) in the cotyledons and embryos will be estimated according to the method of Jaworski (1971). Biological samples will be cut into small pieces of which 200 mg will be weighed and transferred to polythene vials (25 ml), 2.5 ml of phosphate buffer (appendix 1.1), 0.5 ml of 0.2M potassium nitrate (appendix 1.2) solution and 2.5 ml of 5% isopropanol will be added. At least two drops of chloramphenicol will also be added to check bacterial growth in the test sample. These vials will be incubated in a BOD incubator at 30°C for 4 hours in the dark.

3.8.1.1 Colour Development :

In a test tube 0.4 ml of test extract and 0.3 ml each of sulphanilamide and nephthyl ethylene diamine dihydrochloride acid (NED-HCl, appendix 1.3, 1.4) will be added. A pink colour will develop. The sample will be left for 20-30 minutes for maximum colour development, after which it will be diluted

upto 5 ml with double distilled water and sample will read at 540 nm using "Spectronic-20" colorimeter. It will be caliberated for 100% transmittance by using a blank consisting of 4.4 ml water and 0.3 ml each of sulphanilamide and NED-HCl.

Standard curve will be plotted by using graded concentrations of potassium nitrite. Optical density of the test extract will be compared with the help of the caliberated curve and NRA will be calculated in terms of n moles NO_2^- g^{-1}.

3.8.2 Catalase and Peroxidase Activity :

Catalase and peroxidase activity will be determined by using the procedure adopted by chance and Maehly (1955).

3.8.2.1 Extraction :

200 mg sample will be homogenised in 10 ml of 0.1M phosphate buffer, pH 6.8 (appendix 2.1) and centrifuged at 17000 rpm at 2°C for 15 minutes. The clear supernatant will be used as a source of catalase and peroxidase enzymes.

3.8.2.2 Catalase assay :

5 ml of the assay mixture for the catalase activity will contain 300 µ moles of phosphate buffer, pH 6.8, 100 µ moles of H_2O_2 (30%) and 1 ml of the enzyme extract diluted two times. The test tube will be incubated in a BOD incubator at 25°C for 1 minute. The reaction will then be stopped by the addition of 10 ml of 2% H_2SO_4 (v/v) and the residual H_2O_2

will be titrated against 0.01N $KMnO_4$ (appendix 2.2) until faint purple colour persist for at least 15 sec.

A control set in which the enzyme activity will be stopped at 'zero' time, will also be run simultaneously one unit of catalase activity will be defined as that amount of enzyme which breaks 1 µ mole of H_2O_2 per minute under the assay conditions.

3.8.2.3 Peroxidase assay :

5 ml of the assay mixture for the peroxidase activity will contain 125 µ moles of 0.1M phosphate buffer, pH 6.8 (appendix 2.1) 50 µ moles of pyrogallol (appendix 3.1), 50 µ moles of H_2O_2 (30%) and 1 ml of the enzyme extract diluted 20 times. It will be incubated in a BOD incubator at 25°C for 5 minute after which the reaction will be stopped by adding 0.5 ml of 5% H_2SO_4 (v/v). Light orange colour will develop. The amount of purpurogallin formed will be determined by reading the absorbance at 420 nm using "Spectronic-20" colorimeter. A calibrated standard curve will be plotted by taking graded concentrations of pure purpurogallin.

3.8.3 Estimation of auxin-like substances :

This will be done by using the split pea-stem curvature test.

3.8.3.1 Standard auxin-solution :

20 mg of indole-3-acetic acid will be dissolved in 2 ml of 0.1N sodium hydroxide solution in a 100 ml volumetric flask. To this will be added about 80 ml of double-distilled water and the pH neutralised by the addition of 0.1N hydrochloric acid. The volume will finally be made upto 100 ml with water. Graded concentrations of this standard solution will be used to plot a standard curve.

3.8.3.2 Split pea-stem curvature test :

Kent and Gorther (1951) will be followed for this analysis.

3.8.3.3 Raising of seedlings :

Surface sterilized pea seeds will be soaked in water for about 24 hour to ensure seed viability. The seedlings will be raised in acid-washed sand in plastic trays and placed in BOD incubator at $27\pm2^{o}C$ in continuous dark. Complete nutrient solution will be given to the seedlings as and when required. Blue-green light will be used during inspection. From the seventh day onwards, red-light exposure will be given daily for 20-40 minute. Harvesting will be done in red light when the 4th internode is about 5 mm long. The stem will be splitted by making a longitudinal cut through the upper part of the internode just below the first bud.

3.8.3.4 Extraction :

Auxin-like substances will be extracted from the test material according to the method of Sircar and Das (1954).

0.75 g of sample material will be ground finely in a glass mortar for 3 minute with 5 ml of water and an equal quantity of specially cleaned sand. The thoroughly ground material will be carefully transferred to a 25 ml pyrex-glass centrifuge tube, the residue remaining in the motrar will be transferred to the tube by three washings with 10 ml of water. The material will be centrifuged at 4000 rpm for 15 minute. The supernatant will be transferred to a measuring flask and will be suitably diluted and assayed for auxin activity.

3.8.3.5 Measurement of Pea-curvature :

From the harvested seedlings (page 46) 5 to 10 split-stems of 3.5 cm size each having a 3 cm longitudinal cut, will be placed in petriplates with 20 ml of test solution for 24 hours in the dark. These split sections are expected to show two types of curvatures depending on the concentrations of the auxin-like substances in the test solutions (a) outward curvature which is regarded as negative angle produced at the lower concentrations of auxin (b) inward curvature which is considered as positive angle produced at the higher concentrations of auxin.

Outward curvature will be measured as the angle (β) between the unsplit part of the section and the tangent at the extreme tip and at the point of inflection of the lower part of the arm.

Relative auxin-like substances will be calculated with the help of the calibrated curve, plotted by using graded concentration of indole-3-acetic acid.

3.8.4 Estimation of Nitrate :

The nitrate content will be estimated following the method of Johnson and Ulrich (1950).

3.8.4.1 Preparation of powder :

Plant sample will be dried, over night, in an oven at 80°C. These samples will be grinded in an electric grinder and the powder passed through a 72 mesh screen and stored in polythene bags for the analysis of nitrate. Each sample, before analysis, will again be dried over night in an oven at 80°C on a clean sheet of paper. In the morning the samples will be placed in a dessicator for 15 minute for cooling under dry conditions.

3.8.4.2 Extraction and colour development :

50 mg of the above powder will be weighed and transferred to a dried centrifuge tube 25 ml with the addition of 400 mg of calcium sulphate and 12.5 ml of double distilled

water. The sample will be centrifuged for 10 minute at 6000 rpm. The supernatant will be transferred to a 50 ml conical flask containing 1 ml of 0.5% calcium carbonate suspension. The excess solution will be evaporated at water-bath leaving the final volume to 5 ml only. 0.5 ml of H_2O_2 (30%) will be added to the above solution and the flask will be closed with a lid. The lid will be removed and the solution in the flask will be heated further to dryness in a water-bath in order to remove the peroxide. The flask will cooled and 1.25 ml of phenol-di-sulphonic acid (appendix 4.1) will be rapidly added with continuous stirring, 35 ml of distilled water will also added to this solution. Lastly, 3 ml of 50% ammonium hydroxide solution will be pipetted into it, yellow colour developed. The transmittance will be noted, after 15 minute at 400 nm using a "Spectronic-20" colorimeter. A standard curve was plotted using the graded concentrations of standard nitrate solution.

3.8.5 Estimation of Proteins :

Protein level in the samples will be determined by following the method of Lowry et al. (1951).

3.8.5.1 Extraction of soluble and insoluble proteins :

50 mg of the fresh plant material will be transferred to a mortar and grounded with the addition of 5 ml of distilled water and collected in a centrifuge tube with atleast

two washings. The sample will be centrifuged at 4000 rpm for 10 minute. The supernatant will be collected in a 25 ml volumetric flask together with two washings of the residue with distilled water. The volume will be made upto the mark with distilled water and preserved for the estimation of soluble proteins.

To the above residue, 5 ml of 5% TCA (trichloro acetic acid) will be added and allowed to stand, at room temperature for 30 minute with thorough shaking. It will be then centrifuged at 4000 rpm for 10 min and the supernatant discarded. 5 ml of 1N sodium hydroxide will be thoroughly mixed with the residue and allowed to stand in a water-bath at 80^{o}C for 30 minute. The solution will be allowed to cool and centrifuged at 4000 rpm. The supernatant together with three washings with 1N sodium hydroxide will be collected in 25 ml volumetric flask. The volume will be made upto the mark with 1N sodium hydroxide and used for the estimation of insoluble protein.

3.8.5.2 Colour development of soluble protein :

One ml of the above water extract will be transferred to a 10 ml test tube and 5 ml of Reagent C (appendix 7.3) will be added to it. The solution will be mixed well and allowed to stand for 10 minute at room temperature, 0.5 ml of Reagent E (appendix 7.5) will be added rapidly with thorough mixing. After 30 minute blue coloured solution will be transferred

to a colorimetric tube and the per cent transmittance will be noted as 660 nm on a "Spectronic-20" colorimeter. A blank will be run with each sample. The soluble protein content will be calculated by comparing the optical density of each sample with a calibration curve plotted by taking known dilutions of a standard solution of egg-albumin.

3.8.5.3 Colour development of insoluble protein :

One ml of the sodium hydroxide extract will be transferred to a 10 ml test tube and 5 ml of Reagent D (appendix 7.4) will be added to it. The solution will be mixed well and allowed to stand for 10 minute at room temperature 0.5 ml of Reagent E (appendix 7.5) will be added rapidly with immediate mixing. After a lag of about 30 minutes the measurement of the intensity of the colour and other calculations were made in the same way as in soluble protein.

3.8.6. Estimation of Carbohydrates :

Carbohydrate content in the plant material will be estimated as follows :

3.8.6.1 Preparation of powder :

The plant samples will be dried, overnight, in an oven run at 80^{o}C. Each sample will be ground to a fine powder in an electric grinder and passed through 72 mesh screen.

3.8.6.2 Extraction and colour development :

The carbohydrates will be extracted by the method of Yih and Clark (1965) and estimated following the method of Dubois et al. (1956).

Sufficient amount of powder will be sprayed on a clean sheet of paper and dried overnight in an oven running at 80°C. These dried samples will be cooled in a dessicator for about 10 minute before weighing.

50 mg of each sample will be weighed and transferred to a 20 ml glass centrifuge tube. Each tube, 5 ml of 1.5N sulphuric acid (appendix 6.1) will be added. Digestion of the powder will be completed by keeping the tube in a water-bath for 2 hour at 90-95°C. The digested sample will be centrifuged at 4000rpm and the supernatant collected in a 25 ml volumetric flask with two washing and the final volume made up with water. One ml of this extract will be diluted as required, depending upon the plant organ being analysed. One ml of this diluted sample will be taken in a test tube to which one ml of water and one ml of 5% aqueous phenol solution (appendix 6.2) will be added. The test tube will be placed in a beaker containing chilled water and 5 ml of concentrated sulphuric acid will be pipetted quickly into it. After half an hour, the pink coloured solution will be transferred to a colorimetric tube and transmittance noted at 490 nm using a "Spectronic-20" colorimeter. A blank will be

run simultaneously with each set of samples. Calibration curve will be obtained by using known dilutions of a standard aqueous solution of glucose.

3.8.7 Estimation of chlorophyll pigments :

Total chlorophyll pigments will be estimated according to the method of Mackinney (1941).

One gram of the fresh seed material will be ground in a mortar in the presence of sufficient amount of 80% acetone. The extract will be filtered and supernatant collected in the volumetric flask. The residue will be washed repeatedly and each time the washing collected with the supernatant. Finally the volume of the extract will be made up with 80% acetone upto 10 ml. The transmittance of the extracted solution will be directly read at 663 nm, and 645 nm on a "Spectronic-20" colorimeter. The values of optical density (O.D.) in each case will be substituted in the following formula.

$$\text{Total chlorophyll mg/g} = 20.2\ (D\ 645) + 8.02\ (D\ 663) \times \frac{V}{1000 \times W}$$

where V = volume of the solution in ml

W = weight of the seeds in gm.

20.2 and 8.02 are dilution factors.

3.8.8 Estimation of NPK :

The following methodology will be followed for the

estimation of nitrogen, phosphorus and potassium in plant samples.

3.8.8.1 Digestion of powder :

100 mg dry powder of the sample will be taken in a 50 ml Kjeldhal flask. To, this 2 ml of concentrated sulphuric acid (AR) will be added and the mixture heated, on a digestion rack, for 2 hour. The contents are expected to turn black in this duration. After cooling for 15 minute, 0.5 ml of chemically pure H_2O_2 (30%) will be added drop by drop and this procedure will be repeated till a clear solution obtain. The peroxide digested material will be transferred to a 100 ml volumetric flask with at least three washing with distilled water and the volume made upto the mark.

3.8.8.2 Colour development of nitrogen :

Nitrogen will be estimated following the method of Linder (1944). 10 ml aliquot of the peroxide digested material will be taken in a 50 ml volumetric flask to which 2 ml of 2.5N sodium hydroxide and 1 ml of 10% sodium silicate solution will be added to neutralise excess of acid and to prevent turbidity, respectively. The volume of the solution will be made upto the mark with distilled water. In a 10 ml graduated test tube, 5 ml of this solution will be pipetted to which 0.5 ml Nessler's reagent (SQ) will be added.

The final volume will be made upto 10 ml with distilled water. After waiting for 5 minute for maximum colour development, the transmittance of the solution will be determined at 525 nm on "Spectronic 20" colorimeter. A blank consisting of distilled water and Nessler's reagent will be run simultaneously with each sample. A standard curve, taking known dilutions of a standard ammonium sulphate solution, will be plotted. The reading of each sample will be compared with this calibration curve and nitrogen will be expressed in terms of percentage on dry weight basis.

3.8.8.3 Colour development of phosphorus :

Phosphorus content in the sulphuric acid peroxide digested material (page 54) will be estimated by the method of Fiske and Subba Row (1925). 5 ml of the aliquot will be taken in a 10 ml graduated test tube and 1 ml of molybdic acid (appendix 5.2) will be added carefully followed with the addition of 0.4 ml of 1-amino-2-naphthol-4-sulphonic acid (appendix 5.3). Blue colour will develop. Distilled water will be added to make up the volume to 10 ml. The optical density of the solution will be read, after 5 minutes at 620 nm on a "Spectronic-20" colorimeter. A blank will be run simultaneously with each determination. Standard curve will be prepared by using known concentration of mono-basic potassium phosphate solution. The optical density of each sample will be compared with this curve and phosphorus

content will be computed interms of percentage on dry weight basis.

3.8.8.4 Estimation of potassium :

Potassium content in the digested aliquot (page 54) will be directly estimated flame photometrically following the procedure explained by Singh (1988) by using potassium filter. A blank will be run side by side. The reading will be compared with a calibration curve plotted by using known dilutions of a standard potassium chloride solution.

3.8.9 Estimation of calcium and sodium :

Calcium and sodium will be estimated following the procedure given by Singh (1988).

3.8.9.1 Digestion of plant material :

50 mg of the oven dried plant material will be taken in a 50 ml volumetric flask. 2 ml concentrated nitric acid will be poured into it and heated on a hot plate till brown effervescence will be produced.At this stage, sufficient quantity of "TAM" solution will be added to make the solution clear and then heated to dryness. Sufficient quantity of double distilled water will be added and the solution transferred to another 50 ml volumetric flask with three washings. The final volume will be made upto the mark with the addition of double distilled water. This digested material will be used for the estimation of both calcium and sodium.

3.8.9.2 Estimation of calcium :

Flame photometer will be used to read the calcium content in the digested samples with the help of calcium filter. Per cent quantity of the element will be calculated by comparing the readings on a standard curve plotted by using known dilutions of calcium carbonate.

3.8.9.3 Estimation of sodium :

Flame photometer will be used to read the sodium content in the digested samples with the help of sodium filter. Per cent quantity of the element will be calculated by comparing the readings on a standard curve plotted by using known dilutions of sodium bicarbonate.

3.9 Statistical analysis

The experimental data will be analysed statistically following the procedures explained by Panse and Sukhatme (1985). In applying the 'f' tests, the error due to replicates will also be determined. If 'f' value will be found to be significant, at the 5 per cent level of probability, critical difference (C.D.) will be calculated.

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APPENDIX

APPENDIX

PREPARATION OF REAGENTS

The various reagents used for biochemical determinations were prepared according to the following methods.

1.0 Reagents for the estimation of nitrate reductase activity

1.1 Phosphate buffer (pH 7.5)

(a) 13.6 g potassium dihydrogen orthophosphate (KH_2PO_4) was dissolved in sufficient double distilled water and the final volume was adjusted upto 1 litre.

(b) 17.42 g dipotassium monohydrogen orthophosphate (K_2HPO_4) was dissolved in sufficient double distilled water and the final volume was made upto 1 litre.

(c) 160 ml of solution (a) and 840 ml of solution (b) was mixed in order to get pH 7.5.

1.2 Potassium nitrate (0.2M)

2.02 g potassium nitrate was dissolved in enough double distilled water and the final volume was made upto 100 ml.

1.3 Sulphanilamide solution (1%)

1 g sulphanilamide powder was dissolved in 100 ml 3N HCl.

1.4 NED-HCl solution (0.02%)

20 mg NED-HCl N-1-(naphthyl)-ethylene diamine dihydrochloric acid was dissolved in 100 ml double distilled water.

2.0 Reagents for the determination of catalase activity

2.1 Phosphate buffer (pH 6.8)

Stock solutions of KH_2PO_4 and K_2HPO_4 were prepared as given earlier in the method of NR assay. 49 ml of K_2HPO_4 solution was mixed with 51 ml KH_2PO_4 solution. Final volume, 200 ml was made with double distilled water.

3.0 Reagents for the estimation of Peroxidase

3.1 Pyrogallol (50 µM)

63 mg of pyrogallol dissolved in 100 ml of double distilled water. One ml of this is diluted to 100 ml. Final solution will be of 50 µM.

4.0 Reagents for the estimation of nitrate

4.1 Phenoldisulphonic acid reagent

25 g pure white phenol (AR) was dissolved in 150 ml of pure concentrated sulphuric acid to which 75 ml fuming sulphuric acid was added (13% SO_3). The solution was heated for about 2 hours at 100°C in water-bath and cooled. This solution was kept in a dark bottle in refrigerator.

5.0 Reagents for the determination of N and P

5.1 Nessler's reagent

(a) 3.5 g potassium iodide was dissolved in 100 ml double

distilled water to which 4% mercuric chloride was mixed with continued stirring till a slight red precipitate remained (about 325 ml of the solution was required).

(b) 120 g of sodium hydroxide was dissolved in double distilled water and final volume made upto 250 ml.

(c) Solutions (a) and (b) were mixed together and diluted to 1 litre with double distilled water. The solution was stored in an amber coloured bottle in refrigerator.

5.2 Molybdic acid reagent (2.5%)

6.25 g ammonium molybdate was dissolved in 75 ml 10N H_2SO_4. To this solution, 175 ml double distilled water was added in order to get 250 ml of the above reagent.

5.3 Aminonaphthol sulphonic acid

0.5 g 1-amino-2-naphthol-4-sulphonic acid was dissolved in 195 ml 15% sodium bisulphite solution to which 5 ml of 20% sodium sulphite solution was added. The above solution was stored in a dark coloured bottle.

6.0 Reagents for the estimation of carbohydrate

6.1 Sulphuric acid (1.5N)

10.20 ml pure sulphuric acid (AR) was added to enough double distilled water and final volume was made upto 250 ml.

6.2 Phenol (5%)

5 ml distilled phenol was mixed with 95 ml double distilled water.

7.0 Reagents for the estimation of protein

7.1 Reagent A

2% sodium carbonate was mixed with 0.1N sodium hydroxide (1:1).

7.2 Reagent B

0.5% copper sulphate was added to 1% sodium tartrate (1:1).

7.3 Reagent C (alkaline copper sulphate solution)

It was prepared by mixing 50 ml reagent 'A' with 1 ml reagent 'B'.

7.4 Reagent D (carbonate-copper sulphate solution)

Same as reagent 'C', except for the omission of sodium hydroxide.

7.5 Reagent E (Folin's phenol reagent)

100 g sodium tungstate and 25 g sodium molybdate was dissolved in 700 ml distilled water in which 50 ml of 85% phosphoric acid and 100 ml concentrated hydrochloric acid was mixed. The flask was connected with a reflux condenser and boiled gently on a heating mantle for 10 hours. At the

end of the boiling period, 150 g lithium sulphate, 50 ml double distilled water and 3-4 drops of liquid bromine was added to this flask. The reflux condenser was removed and the solution in the flask was boiled for 15 minutes in order to remove excess bromine, cooled and diluted to 1 litre.

The strength of this acidic solution (1N) was tested by treating it with 1N sodium hydroxide using phenolphthalein as an indicator.

8.0 Reagent for calcium and sodium

8.1 TAM solution (tri acid mixture)

It is a mixture of three acids like nitric acid, sulphuric acid and perchloric acid in the ratio of 10:5:4.